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Full Length Research Paper

In vitro anti-*Leishmania amazonensis* activity of the polymeric procyanidin-rich aqueous extract from *Syagrus coronata*

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***Leishmania amazonensis* is one of the major etiological agents of diffuse cutaneous leishmaniasis, which is frequently unresponsive to all known treatments. *Syagrus coronata* (Arecaceae) is a palm tree with industrial applications used in local medicine by the Brazilian "*caatinga*" communities. This work evaluates the *in vitro* leishmanicidal activity of *S. coronata* on *L. amazonensis*. Promastigote forms of *L. amazonensis* were treated with different concentrations of the aqueous extract from *S. coronata*. In addition, we evaluated the effect of the aqueous extract on infection of mouse peritoneal macrophages and nitric oxide production. The minimal inhibitory concentration (MIC) of the aqueous extract of *S. coronata* was 8.3 µg/ml. Morphological changes in *L. amazonensis* promastigotes treated with 50 µg/ml of the aqueous extract were observed by light microscopy. Pretreatment of mouse peritoneal macrophages with 33 µg/ml of *S. coronata* aqueous extract reduced the association index between macrophages and *L. amazonensis* by 70.4%, with a concomitant increase of 158.3% in nitric oxide production by the infected macrophages. In addition, the aqueous extract exhibited no cytotoxic effect on mammalian cells and elicited no allergic reactions *in vivo*, indicating good prospects for the development of new drugs of herbal origins to treat leishmaniasis.**

Key words: Antileishmanial activity, crude extracts, nitric oxide, polyphenols, semi-arid vegetation.

INTRODUCTION

Leishmaniasis is a tropical disease caused by species of the *Leishmania* genus that are spread throughout Africa, Asia, Europe, North America and South America, with an estimated 12 million people infected worldwide and a global incidence of 2 million new cases annually (CDC, 2011). Clinically, leishmaniasis occurs in visceral, cutaneous and mucocutaneous forms, with 90% of the latter present in Afghanistan, Saudi Arabia, Algeria, Brazil, Iran, Iraq, Syria and Sudan (WHO, 2010). The New World form of the disease is mainly transmitted by flies of the genus *Lutzomyia* (Ashford, 2000). Upon inoculation of the host, promastigotes are phagocytized by skin macrophages, where they transform into ovoid bodies known as amastigotes. In mammals, *Leishmania* are dimorphic obligate intracellular parasites that infect and multiply primarily within the phagolysosomal

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compartment of mononuclear phagocytes (Gonçalves et al., 2005). Thus, the pathogenesis of *Leishmania* infection depends to a large extent on the relationship between the parasite and host macrophages.

The pentavalent antimonials, which are the recommended drugs for the treatment of cutaneous leishmaniasis, were first introduced 60 years ago; however, serious side effects and the emergence of resistant strains limit their effectiveness (Croft et al., 2006a, b). In addition, these drugs require parenteral administration and are toxic and expensive, with a restricted therapeutic spectrum for all clinical forms of leishmaniasis (Davis et al., 2004). The lack of an effective leishmanicidal drug has evoked a renewed interest in medicinal plants as sources of new chemotherapeutic compounds that are more effective and have fewer side effects (Tiuman et al., 2005).

In every country, traditional medicines are ingrained in magical or religious beliefs or in popular experience, and the World Health Organization (WHO) is currently devising guidelines for clinical research methodologies and appraisal of the effectiveness of traditional medicines (Firenzuoli and Gori, 2007). Brazil not only has one of the world's highest degree of biodiversity, but it also has an enormous cultural diversity and an as yet under-utilized repertoire of plants with a potential economic value (Albuquerque et al., 2007). The "*caatinga*" (Brazilian semi-arid) possess ecological characteristics that are well known to many arid regions, including low relative humidity, high temperatures and by rather unpredictable seasonality of rains. The "*caatinga"* vegetation is a highly threatened biome covering a vast area in northeastern Brazil and is the source of many little-studied natural resources (Araújo et al., 2007). Many medicinal plant species from the "*caatinga"* are widely known and used in folk medicine and for commercial manufacturing of phytotherapeutic products, including the following: *Myracrodruon urundeuva* Allemão, *Amburana cearensis* (Arr. Cam.) A.C. Smith, *Erythrina velutina* Willd., *Anadenanthera colubrina* (Vell.) Brenan var. *cebil* (Griseb) Altschul, and *Sideroxylon obtusifolium* (Roem. and Schult.) T.D. Penn (Albuquerque and Oliveira, 2007). *Syagrus coronata* (C. Martius) Becc. (Palmae) is a palm tree of South American distribution. In Brazil, this palm tree appears mainly along the coastal area of the state of Bahia and grows to approximately 10 m tall, with a crown of arching, semi-plumose leaves. The "licuzeiro", as *S. coronata* is popularly known, is an economically important palm tree grown as an ornamental, for the oil extracted from its seeds and for the wax from its leaves, which has several industrial applications (Lins et al., 2002). The flowers and fruits of *S. coronata* are used in folk medicine to treat dysentery and cataracts (Albuquerque et al., 2007); however, at present, there are no scientific reports regarding the pharmacological activity of *S. coronata*. Thus, the aim of the present study was to investigate the activity of an aqueous extract of *S. coronata* against *L. amazonensis* parasites and its effects on macrophage infection and nitric oxide (NO) production.

MATERIALS AND METHODS

Plant material

Syagrus coronata coconut fruits, commonly known as "*licuri*", were collected in Santa Luz, state of Bahia, Brazil (Long.: -39:21:52.89; Lat.: -11:14:35.67; height above sea level: 370 m) and authenticated by Dr. Larry Noblick, Herbário da Universidade Estadual de Feira de Santana, Bahia (Brazil), where a voucher specimen is deposited (HUEFS n. 63069).

Polyphenolic extract

The wet coconut husk (20 g) was finely ground and soaked for 1 h in 300 ml of boiling distilled water. The extract was filtered and lyophilized, yielding 1.3 g of crude aqueous extract. The extract was then dissolved in RPMI medium at 1 mg/ml dilution and sterilized by filtration using a 0.22 µm Millipore membrane prior to use in leishmanicidal assays. This *S. coronata* crude aqueous extract, prepared as described, was used for all assays throughout this study.

Characterization of procyanidins

The crude aqueous extract was subjected to HPLC/DAD analysis, according to a protocol devised by Peng et al. (2001) to analyze procyanidins in grape seeds. Samples (20 µl) were loaded onto a 4.6 x 250 mm C_{18} column (Exsil 100 5 µ ODS, Activon, Sydney, Australia) with a C_{18} guard cartridge with the same packing material equilibrated in solvent A [0.2% (v/v) phosphoric acid]. Phenolic compounds were eluted with a gradient of solvent B [82% (v/v) acetonitrile, 0.04% (v/v) phosphoric acid] as follows: 0 to 15% solvent B in the first 15 min, 15 to 16% from 15 to 40 min, 16 to 17% from 40 to 45 min, 17 to 43% from 45 to 48 min, 43 to 52% from 48 to 49 min, held isocratic at 52% from 49 to 56 min, reduced from 52 to 43% from 56 to 57 min, and finally reduced from 17 to 0% from 58 to 60 min. Peaks were detected at 280 nm and identified by comparison with the retention times of standards.

The concentration of procyanidins in the sample solution of *S. coronata* lyophilized crude aqueous extract was determined by the vanillin-HCl assay (Nakamura et al., 2003). Briefly, 2.5 ml of methanol (control) or 1% vanillin solution in methanol (sample) and 2.5 ml of 9 M HCl in methanol was added to a test tube containing 1 ml of catechin solution (0 to 300 µg/ml in methanol) or test solution (150 to 250 µg/ml polyphenols in methanol). The reaction mixture was incubated for 20 min at 30°C, and the absorbance at 500 nm was measured. The absorbance was calculated as follows for each standard and sample solution: a calibration curve was prepared using the calculated absorbance for the catechin solution, and the total procyanidin in each test solution was calculated from the calibration curve.

Parasite culture

Promastigote forms of *Leishmania amazonensis* (Raimundo strain MHOM/BR/76/Ma–5) were maintained by weekly subculturing in PBHIL medium (Rodrigues et al., 2010) supplemented with 10% fetal bovine serum (FBS), at 26°C. Infectivity of the parasites was maintained by periodic hamster footpad inoculation.

Leishmanicidal activity

Promastigotes of *L. amazonensis* (10^6 parasites/ml) were incubated in RPMI medium in the absence or presence of 50 µg/ml of crude aqueous extract from the coconut husk of *S. coronata* at 37°C, and parasite survival and cell morphology were evaluated by optical microscopy at 10 min intervals. Parasite viability was assessed before and after incubation by motility and by trypan blue exclusion, using a hemocytometer chamber.

Cell viability was determined using the following formula: [100 – (L2 / L1)]×100, where L1 is the percentage of viable control cells and L2 is the percentage of viable treated cells, as previously described (Delorenzi, 2001). Alternatively, after 30 min of parasite growth in the presence of the *S. coronata* coconut husk aqueous extract (16 µg/ml), cells were centrifuged and washed three times in PBS prior to resuspension in new culture medium without the plant extract to evaluate the leishmanicidal or leishmanistatic effect. Growth was determined by counting the cells over a 120 h period of incubation *in vitro*.

Minimum inhibitory concentration (MIC) evaluation

L. amazonensis promastigotes (10^6 parasites/ml) were incubated in fresh medium (PBHIL) supplemented with 10% FBS in the absence or presence of several concentrations (1 µg/ml to 1 mg/ml) of aqueous extract from the coconut husk of *S. coronata* at 26°C for 120 h (cell growth was determined daily by assessment of visible turbidity). MIC was considered the lowest concentration of the aqueous extract that prevented the growth of *L. amazonensis in vitro*. Parasite growth was determined daily using a hemocytometer chamber.

Mouse peritoneal macrophages

Non-elicited peritoneal macrophages from female Swiss mice (6 to 8 weeks of age) were collected in cold PBS (150 mM NaCl; 20 mM phosphate buffer, pH 7.2) and allowed to adhere to coverslips placed in 24 well culture plates for 30 min at 37°C in a 5% CO_2 atmosphere. Non-adherent cells were then removed, and the adhered macrophages were washed twice with PBS and cultured for 24 h in culture medium (RPMI) (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% FBS.

Infection of macrophages and nitric oxide production

Mouse peritoneal macrophages were obtained as described previously. Parasites and/or macrophages were either treated with 16 and 33 µg/ml of aqueous extract from *S. coronata* or untreated for 20 min prior to the macrophage-parasite interactions. Adherent cultured macrophages and free parasites were washed once and resuspended in fresh RPMI culture medium. Intact, live *L. amazonensis* promastigotes were then added to macrophages in culture plates. Parasite-macrophage interactions were conducted at 37°C for 90 min using parasites and/or macrophages pretreated with the aqueous extract. An MOI of 10 was used for all infection assays.

After interaction assays were completed, coverslips were fixed and Giemsa stained, and the percentage of infected macrophages was determined by counting 600 cells on triplicate coverslips. The association indices were determined by multiplying the percentage of infected macrophages by the mean number of parasites per infected cell. The association index was considered to be the number of parasites that successfully infected the macrophages. The supernatants from control (macrophages in culture only) and *L. amazonensis*–infected macrophages were analyzed for their nitrite content by the Griess reaction as previously described (Grynberg et al., 1999). The absorbance at 550 nm was measured, and the concentration of nitrite was calculated using a linear regression of a standard curve. To obtain mouse peritoneal macrophages both for infections with *Leishmania* and for nitric oxide measurements, mice were sacrificed according to all federal guidelines and institutional policies.

Anti-intracellular amastigote activity

To evaluate the effect of the aqueous extract on intracellular amastigotes, macrophages were infected with *L. amazonensis* promastigotes (logarithmic growth phase) at a multiplicity of infection (MOI) of 10 and incubated for 90 min at 37°C in 5% CO_2. Next, free promastigotes were removed by extensive washing with PBS, and the infected macrophages were then incubated for 24 h to allow for complete promastigote differentiation into intracellular amastigotes. The infected macrophages were then treated with the aqueous extract (16 and 33 µg/ml) for 20 min. After treatment, culture supernatants were collected for NO analysis, and coverslips were fixed as described above.

Cytotoxicity assay

The cytotoxicity of the aqueous extract toward macrophages was measured using the neutral red uptake assay. Briefly, macrophages were cultivated in 96 well microtiter plates (150 µl containing 10^5 cells/ml in EAGLE-MEM medium/well) at 37°C in a humidified 5% CO_2 atmosphere. The medium was supplemented with L-glutamine (0.10 g/L), HEPES (2.38 g/L), penicillin G (10^5 IE/L), streptomycin sulfate (0.10 g/L) and 4% FBS. After a 24 h incubation, 50 µl of 33 µg/ml aqueous extract was added to the cultures, and 50 µl of EAGLE-MEM medium was added to the control cells.

After further incubation for 48 h, control and treated cells were washed three times with PBS (pH 7.2), and 100 µl of neutral red solution (0.3% in EAGLE-MEM) was added to each well. After a 3 h incubation at 37°C with the neutral red solution, the cells were again washed three times with PBS. Finally, 100 µl of a solution of 1% acetic acid and 50% ethanol was added to the wells, and the optical density (OD) of the supernatants was measured at 540 nm.

***In vivo* primary dermal irritation, cumulative dermal irritation and ocular irritation tests**

For each irritation test, 3 Balb/c mice (6 to 8 weeks of age) were used. To perform the dermal tests, we selected 2 different shaved and gently scraped regions (right and left) that were approximately 6 mm in diameter on the back of each mouse. In the primary irritation test, we used a small volume (0.04 ml) of sterile *S. coronata* aqueous extract (100µg/ml). The treatment sites were observed after application of the extract and over the subsequent 7 days. In the cumulative dermal irritation tests, we added 0.5 ml of *S. coronata* extract (100 µg/ml) daily after the first application. For the ocular irritation test, we dropped 0.05 ml of the extract (100 µg/ml) in both eyes and monitored inflammation at 24, 48 and 72 h post-application.

Statistical analysis

All experiments were performed in triplicate. The mean and standard error of at least three experiments were determined. The differences between the mean values obtained for experimental groups was evaluated by the Student's *t* test. *P*-values of 0.05 or less were considered significant.

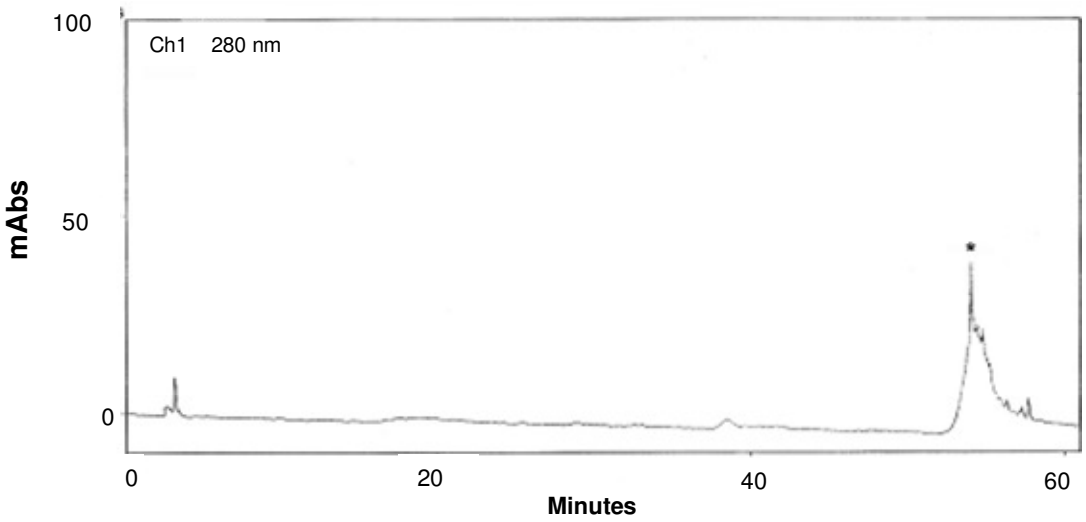

Figure 1. A trace of reverse phase HPLC analysis of *S. coronata* lyophilized crude aqueous extract. The asterisk (✱) in the figure indicates polymeric procyanidins (Rodrigues et al., 2011).

RESULTS

Aqueous extract analysis

The lyophilized crude aqueous extract was analyzed by HPLC. The chromatogram (Figure 1) features a late-eluting broad peak. According to the protocol devised by Peng et al. (2001) for the analysis of tannins, this elution behavior and UV absorption characteristics indicate the presence of polymeric procyanidins. The vanillin-HCl assay is highly specific for procyanidins and involves acidic hydrolysis of the condensed tannins followed by coupling of the flavanol units to vanillin. Polymeric procyanidins were detected at a concentration of 146 mg/g of aqueous extract.

Minimum inhibitory concentration

The lowest concentration of the *S. coronata* crude aqueous extract that abrogated the growth of *L. amazonensis* promastigote forms was 8.3 µg/ml.

Leishmanicidal activity

A time course of the viability of *L. amazonensis* promastigotes and of mouse peritoneal macrophages in the absence or presence of *S. coronata* crude aqueous extract is shown in Figure 2. At a concentration of 50 µg/ml, the *S. coronata* crude aqueous extract was able to kill 100% of *L. amazonensis* promastigotes in 60 min with no toxicity toward mouse macrophages (Figure 2). Increased cellular volume was noted among parasites exposed to the aqueous extract, followed by membrane disruption (Figure 3). In addition, after a 30 min incubation with the aqueous extract, parasites were no longer able to grow in fresh medium.

Infection of macrophages

Figure 4 shows the effects of the crude aqueous extract on the interaction between *L. amazonensis* and macrophages. Before co-culture, parasites and/or mouse peritoneal macrophages were treated with two different concentrations of *S. coronata* crude aqueous extract (16 and 33 µg/ml) for 20 min or left untreated (Figure 4A). The crude aqueous extract was able to reduce the association indices in all systems tested. When macrophages were pretreated with 16 or 33 µg/ml of the crude aqueous extract and incubated with untreated parasites, association indices were 37 and 70.4% lower than the controls (untreated macrophages and parasites), respectively. When parasites were pretreated with 16 or 33 µg /ml of the crude aqueous extract and incubated with untreated macrophages, the association indices decreased 21.8 and 54.3% compared to the control system, respectively, similar to the results obtained for the co-culture of pretreated macrophages with pretreated parasites (34.1 and 51.8%, respectively). Finally, when macrophages were infected with *L. amazonensis*, 24 h prior to treatment with 16 or 33 µg/ml of the crude aqueous extract, the association indices were 52.3 and 71% lower than the control, respectively (Figure 4B).

Nitric oxide production

Culture supernatants of mouse peritoneal macrophages

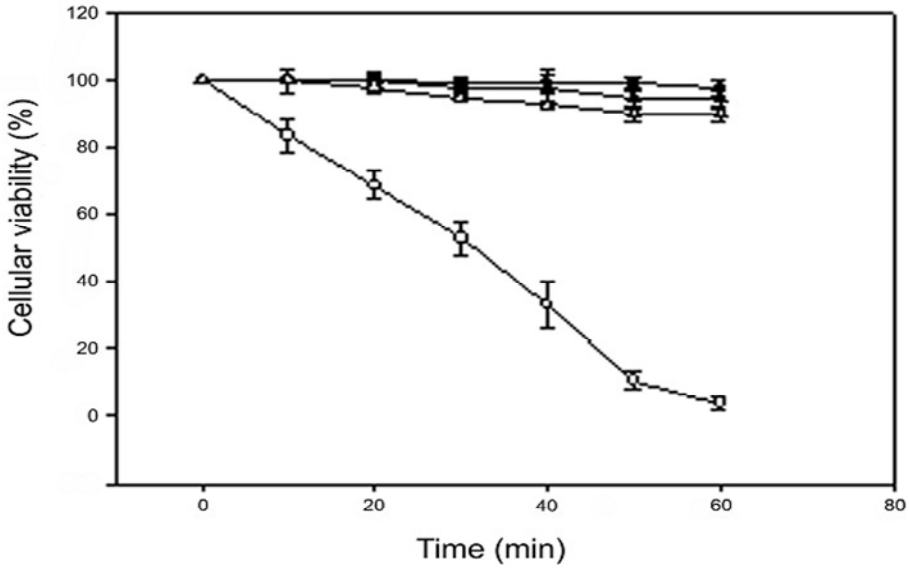

Figure 2. Time course of the viability of promastigote forms of *Leishmania amazonensis* and mouse peritoneal macrophages in the absence or presence of the aqueous extract from *S. coronata*. The viability (%) of parasites and macrophages was calculated as [100 – (L2/L1)] × 100, where L1 is the percentage of viable control cells and L2 is the percentage of viable treated cells. (●) *L. amazonensis* promastigotes (control); (○) *L. amazonensis* promastigotes + *S. coronata* aqueous extract at 50 µg/ml; (▲) mouse peritoneal macrophages (control); (△) mouse peritoneal macrophages + *S. coronata* aqueous extract at 50 µg/ml (Rodrigues et al., 2011).

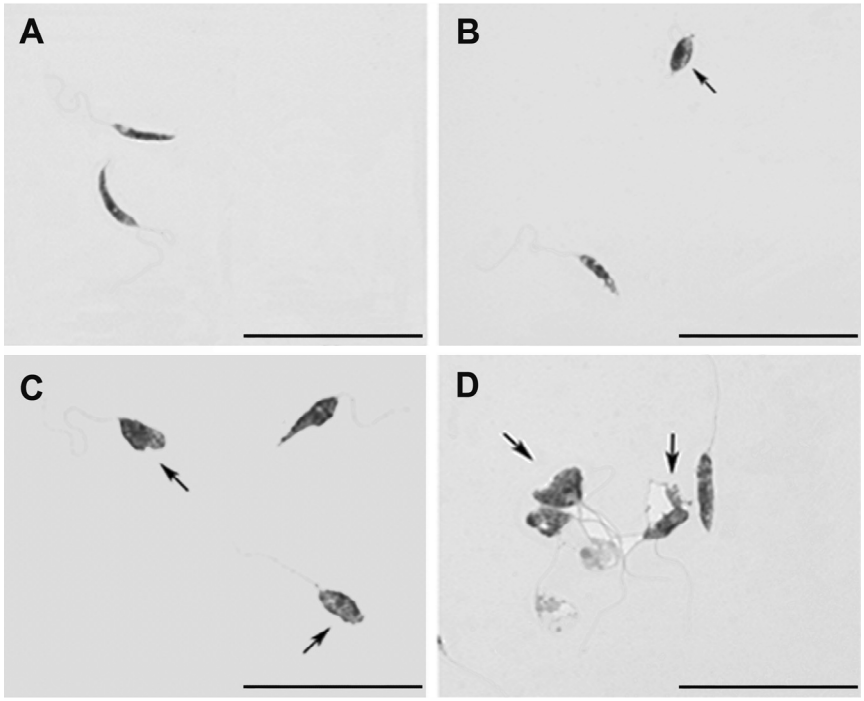

Figure 3. Microscopic observation of the viability time course of *L. amazonensis* promastigote forms incubated with 50 µg/ml *S. coronata* aqueous extract. (A) Control parasites; (B) parasites treated for 20 min; (C) parasites treated for 40 min; (D) parasites treated for 60 min. Note the increase in cell volume (arrows in B and C) and the complete lysis of the parasite cell (D). Scale bar, 20 µm (Rodrigues et al., 2011).

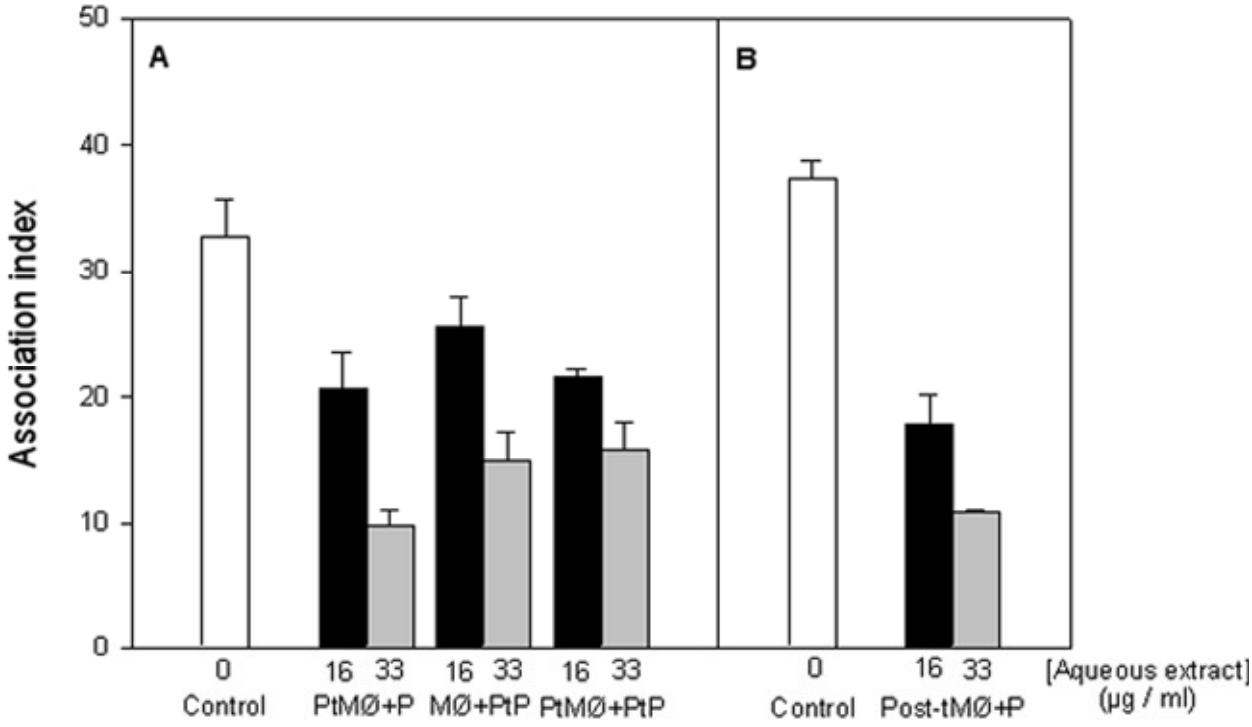

Figure 4. Effects of *S. coronata* aqueous extract on the *Leishmania amazonensis*-macrophage interaction. Parasites and/or mouse peritoneal macrophages were either treated with 16 µg/ml (black bars) or 33 µg/ml (gray bars) of *S. coronata* aqueous extract or untreated (white bar) 20 min prior to the co-culture of macrophages and parasites (A). The adherent cultured macrophages and the free parasites were washed once and resuspended in fresh culture medium. Intact, live *L. amazonensis* promastigotes were then added to the macrophages in culture plate wells. Macrophages previously infected with *L. amazonensis* were treated with the aqueous extract (16 and 33 µg/ml) for 20 min and then incubated for 90 min at 37°C in 5% CO_2 (B). Association indices were determined by light microscopy by counting 600 cells on triplicate coverslips after 90 min of interaction. Each bar represents the mean ± standard error of at least three independent experiments, which were performed in triplicate. Association indices of assays performed using pretreated macrophages and/or pretreated parasites with *S. coronata* extract are significantly different from the association index of control (untreated) macrophages. (MØ) macrophages; (P) parasites; (PtMØ) pretreated macrophages; (PtP) pretreated parasites; (Post-tMØ) post-treated infected macrophages (Rodrigues et al., 2011).

were treated with 16 or 33 µg/ml of the aqueous extract produced 40.6 and 60.1% more nitric oxide than control (uninfected and untreated) macrophages. Infected macrophages produced 80.4% more nitric oxide than uninfected macrophages. When macrophages were pretreated with 16 or 33 µg/ml of the aqueous extract prior to infection, nitric oxide production was 108.3 and 158.3% higher than the control infected macrophages, respectively. When parasites were pretreated with 16 or 33 µg/ml of the aqueous extract, nitric oxide production was 58.3 and 83.3% higher than the untreated infected macrophages, respectively.

The pretreatment of both macrophages and parasites with 16 or 33 µg/ml of the aqueous extract resulted in an increase in nitric oxide production of 27.7 and 83.3%, respectively. When macrophages were infected with *L. amazonensis* 24 h prior to treatment with 16 or 33 µg/ml of the aqueous extract, nitric oxide production was 52.3 and 90.4% higher than the control, respectively (Figure 5B).

In vivo allergy tests

None of the animals tested showed any dermal or ocular allergic reactions to the extract, and as a consequence of these results, the extract was considered non-allergenic.

DISCUSSION

The current treatment regimens for leishmaniasis, which are based on chemotherapy, are limited and are not ideal because they are often associated with severe side effects (Tasdemir et al., 2006). The standard treatment for the disease is the administration of pentavalent antimonials. Traditional alternatives to antimonials used in unresponsive cases are amphotericin B and pentamidine, which cause serious, toxic side-effects (Sereno et al., 2000; Croft et al., 2006a), and the emergence of drug-resistant parasites presents a major problem (Croft et al., 2006b). All of these factors

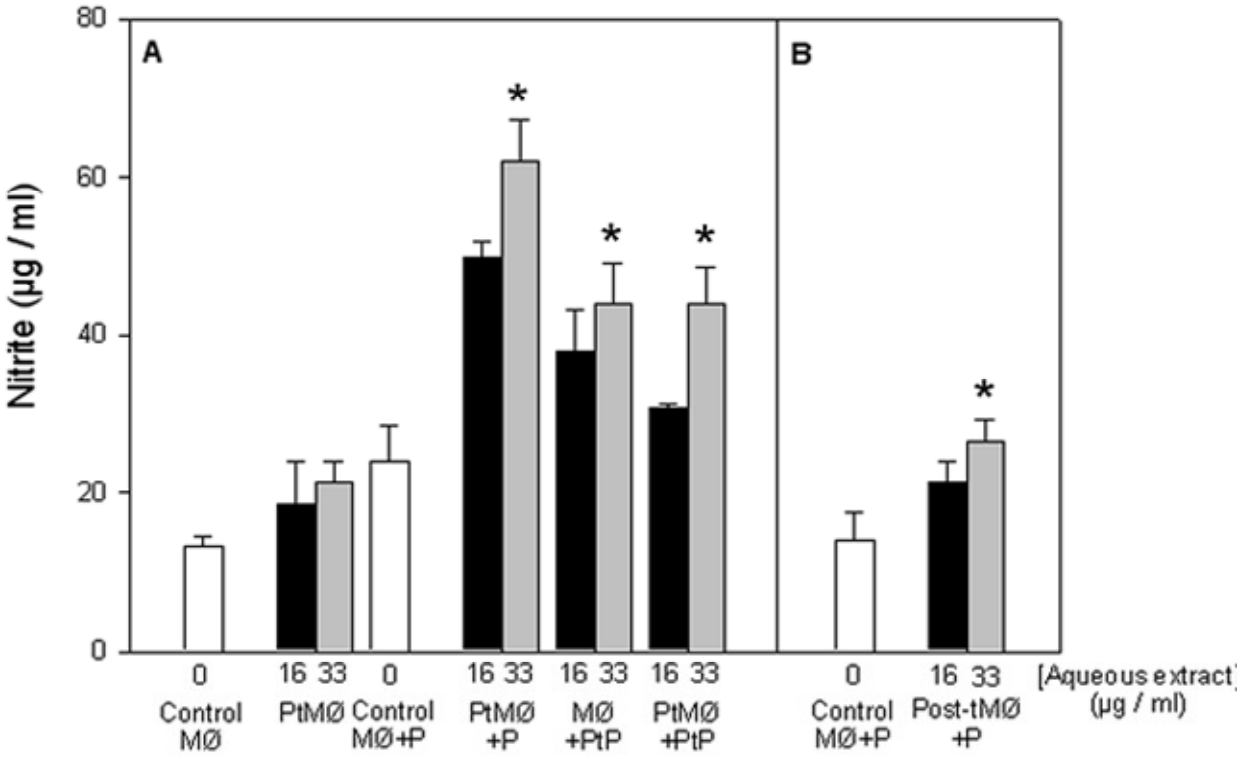

Figure 5. Effects of the *S. coronata* aqueous extract on the nitric oxide production by mouse peritoneal macrophages. Parasites and/or macrophages were either treated with 16 or 33 µg/ml of *S .coronata* aqueous extract 20 min prior to macrophage-parasite co-culture or left untreated. The adherent cultured macrophages and the free parasites were washed once and resuspended in fresh culture medium. Intact, live *L. amazonensis* promastigotes were then added to the macrophages in culture plate wells (A). Macrophages previously infected with *L. amazonensis* were treated with the aqueous extract (16 and 33 µg/ml) for 20 min and then incubated for 90 min at 37°C in 5% CO_2 (B). The supernatants from control and *L. amazonensis*–infected macrophages were collected, and the nitrite concentration of each system was determined by the Griess reaction, as described in Materials and Methods. Each bar represents the mean ± standard error of at least three independent experiments, which were performed in triplicate. The asterisk (✱) indicates a significantly different value from the infected macrophages, in which neither the macrophages nor the parasites were treated with the *S. coronata* aqueous extract. (MØ) macrophages; (P) parasites; (PtMØ) pre-treated macrophages; (PtP) pre-treated parasites; (Post-tMØ) post-treated infected macrophages (Rodrigues et al., 2011).

underscore the urgent need for new, inexpensive, safe and easy-to-administer substances for the treatment of this infectious disease. Traditional medicines have been used since ancient times; they are the accumulation of practices based on the theories, beliefs and experiences of different cultures.

Traditional medicines are used for the maintenance of health and in the prevention, diagnosis, improvement and treatment of illnesses (Firenzuoli and Gori, 2007). Based on traditional medicine, new drugs of herbal origins discovered through ethnopharmacological studies have shown promising results. Studies focusing on herbal remedies useful for the treatment of leishmaniasis have been undertaken in French Guiana (Sauvain et al., 1996), Bolivia (Fournet et al., 1994), Colombia (Weniger et al., 2001), Peru (Kvist et al., 2006) and Brazil (Muzitano et al., 2006). Along these lines, our laboratory has also initiated and developed original studies of alternative compounds for the control of protozoan parasites (Rosa et al., 2003; Mendonça-Filho et al., 2004; Ueda-Nakamura et al., 2006; de Almeida et al., 2007). In the course of screening for leishmanicidal compounds from Brazilian plants, in this work, we identified the novel pharmacological activity of the crude aqueous extract obtained from the *S. coronata* palm.

Here, we showed *in vitro* that a crude aqueous extract at a concentration of 50 µg/ml killed 100% of *L. amazonensis* promastigote forms in a 60 min time course (Figure 2). An increased cellular volume followed by membrane disruption could be noted after 40 min of exposure, as revealed by light microscopy (Figure 3). In a previous study, we described the leishmanicidal activity of a polyphenolic-rich extract from *Cocos nucifera* husk fiber, which, at a concentration of 10 µg/ml (MIC), led to parasite damage after 30 min (Mendonça-Filho et al., 2004). Similar to the present work, parasites also seemed to lose osmotic regulation, resulting in increased cellular volume and lysis. Saleheena et al. (2004) demonstrated

the leishmanicidal activity of an aqueous onion extract (*Allium cepa*) on several strains of *Leishmania* (Saleheena et al., 2004). According to the authors, the aqueous extract at 1.25 mg/ml was found to be leishmanicidal for all of the *Leishmania* strains tested, whereas 50% of the parasites of all strains were killed at an average concentration of 0.376 mg/ml after 72 h of treatment. Here, after a 30 min incubation with *S. coronata* crude aqueous extract at 50 µg/ml, the parasites were no longer able to grow in fresh medium, suggesting irreversible metabolic damage.

We observed that the *S. coronata* crude aqueous extract was able to reduce the association index between mouse peritoneal macrophages and *L. amazonensis,* with a concomitant increase in NO production in all systems tested (Figures 4 and 5). When macrophages were pre-treated with the crude aqueous extract at 33 µg/ml, the association index was reduced approximately 70.4% (Figure 4A), and an increase of 158.3% in NO production compared with the controls was detected (Figure 5A). Pretreatment of parasites with 33 µg/ml crude aqueous extract reduced the association index by 54.3%, and NO production was 83.3% higher than the controls. Tiuman et al. (2005) described the leishmanicidal activity of parthenolide isolated from the hydroalcoholic extract of the aerial parts of *Tanacetum parthenium* and the survival index of intracellular amastigotes was reduced 50% when preinfected macrophages were treated with the purified parthenolide at 0.87 µg/ml.

In our study, the infection of macrophages 24 h prior to treatment with 33 µg/ml crude aqueous extract reduced the association index by 71%, with a concomitant increase of 90.4% in NO production. Our results suggest that this aqueous extract likely induces the synthesis of NO by murine macrophages, which potentially enhances the killing mechanisms of these cells. Lemesre et al. (1997) reported the effects of authentic NO gas, which mimics macrophage-mediated cytotoxicity, on the *in vitro* viability and proliferation of axenically cultured amastigote and promastigote forms of *Leishmania mexicana*, *L. amazonensis*, and *Leishmania chagasi*. In that study, the authors demonstrated that NO action led to lethal metabolic inhibition in both parasite developmental stages by, at least in part, triggering iron loss from enzyme(s) with iron–sulfur prosthetic groups, particularly aconitase. Here, we observed the correlation between association index, NO production and parasite elimination, suggesting that NO could exert an important role in the clearance of infection. A previous report showed that interferon-gamma (IFN-γ) (Gantt et al., 2001) potentiated killing of *L. chagasi* by macrophages via the induction of inducible nitric oxide synthase (iNOS); however, the mechanism by which the crude aqueous extract of *S. coronata* augments NO production is unclear.

In the search for therapeutic agents for the treatment of NO-related diseases, polyphenols have been found to inhibit NO production (Ishii et al., 1999; Cheon et al., 2000). Nevertheless, a number of papers have reported beneficial effects of polyphenols on infectious diseases that may be due to immunomodulatory activities, though the mechanism of action remains to be clarified (Kolodziej and Kiderlen, 2005). The leishmanicidal activities of phenolic compounds against *Leishmania* promastigotes and amastigotes have been described in several works (Mendonça-Filho et al., 2004; Kolodziej and Kiderlen, 2005; Kolodziej et al., 2008).

The polyphenol content of the *S. coronata* aqueous extract was determined, and we observed that polymeric procyanidins (tannins) were found to be the principal components (Figure 1). Kiderlen et al. (2001) demonstrated the leishmanicidal and immunomodulatory activities of a series of 28 polyphenols, which were evaluated for extra- and intracellular leishmanicidal activity and macrophage activation, including the release of NO and tumor necrosis factor (TNF), and for IFN-like properties. The potential of the polyphenols tested to induce NO release was moderate, ranging from 7-54 µM (IFN- γ/LPS 119 µM). Recently, Guerra et al. (2011) described the immunomodulatory activity of an aqueous extract of babassu used as vaccine adjuvant. In that study, the aqueous extract (5 mg) induced the production of IFN-γ and NO by mouse splenocytes *in vitro*. Trun et al. (2006) demonstrated that the aqueous-ethanolic extract of the roots of *Pelargonium sidoides* (Eps® 7630) was capable of significantly enhancing nitric oxide synthase (iNOS) and cytokine mRNA levels in *Leishmania*-infected RAW264.7 cells compared to uninfected cells. The results reported in that study provide significant insights into the mode of action of plants extracts at the molecular level and could partially explain how phenolic compounds, such as polyphenols, influence the immune system.

Conclusion

In conclusion, drug candidates for the treatment of leishmaniasis should demonstrate both a direct and selective action on the parasites, such as stimulation of the host immune system, to increase the likelihood of clearing the infection. The toxicity of the polymeric procyanidin-rich crude aqueous extract toward *L. amazonensis* with concomitant macrophage activation and no allergic reactions *in vivo*, demonstrate the potential use of this plant as a leishmanicidal drug.

Further investigation will be necessary to elucidate the relationship between phenolic compounds and the increased production of NO. The results presented here reveal a novel pharmacological activity for the procyanidin-rich aqueous extract from the coconut husk of *S. coronata*. However, further investigation is necessary to characterize the active compounds in the

aqueous extract, and such studies are currently underway in our laboratory.

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